



(11) **EP 0 855 184 A1**

(12) **EUROPEAN PATENT APPLICATION**

(43) Date of publication:
29.07.1998 Bulletin 1998/31

(51) Int. Cl.⁶: **A61K 39/39**, **A61K 48/00**,
C07K 14/195, **C07K 14/435**

(21) Application number: **97101019.4**

(22) Date of filing: **23.01.1997**

(84) Designated Contracting States:
DE

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Remarks:

The applicant has subsequently filed a sequence listing and declared, that it includes no new matter.

(54) **Pharmaceutical composition comprising a polynucleotide and an antigen especially for vaccination**

(57) The invention discloses a pharmaceutical composition comprising at least one fragment of a polynucleotide and at least one antigen, especially for the preparation of a vaccine.

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Description

The present invention relates to pharmaceutical compositions comprising the introduction of short nucleotide sequence into vertebrates to achieve regulation of growth, induction of cellular transcription and translation, protein synthesis, protein expression or protein secretion and the possible applications thereof. The pharmaceutical compositions are useful in vaccination protocols but also in any other therapeutic situation in which immunomodulation is of benefit, such as sub-optimal immune responses, pathogens, tolerance or autoimmunity.

It is known from the prior art that phosphorothioate oligonucleotides may have an influence on the regulation of gene expression [Bielinska et al., Science, vol. 250 (1990), p. 997-250]. Krieg et al. [Nature, vol. 374 (1995), p. 546-549] report that bacterial DNA may trigger direct B cell activation. Krieg et al. report that bacterial DNA and synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotides induce murine B cells to proliferate and secrete immunoglobulin in vitro and in vivo.

In the course of the present invention it has been surprisingly found that bacterial sequences as described in the prior art may cause severe side effects such as lethal shock. It has been found, however, that other DNA sequences may have a beneficial effect on mammals and may therefore be used for the preparation of pharmaceutical compositions having a beneficial effect on the immune system.

It is an object of the present invention to provide pharmaceutical compositions comprising inexpensive, stable and safe immune adjuvants and immunomodulatory substances for human and animal prophylactic and therapeutic use.

It is known that cells of the immune system are exported from the bone marrow and undergo a series of differentiation events which confer upon them the capacity to recognize and control foreign pathogens and cancer cells by discriminating between self versus non-self. These differentiation and education events are tightly controlled by cell surface receptor engagement via intracellular signal transduction and the milieu of autocrine, paracrine and endocrine soluble ligands, typically referred to as cytokines. Cell to cell interaction occurs in discrete locals such as the thymus, spleen or lymph nodes but also in the periphery. The system thus balances receptor and cytokine input signals to regulate cellular proliferation, differentiation and maturation of immune effector cells [Paul, Cell, 57:521 (1989)]. Through outside intervention the immune system can be manipulated, namely enhanced, e.g. vaccination or cytokine therapies, or suppressed, e.g. drug intervention or cytokine therapies.

The immune system of vertebrates consists of several interacting components. The best characterized and most important parts are the humoral and cellular (cytolytic) branches. Humoral immunity involves antibodies, proteins which are secreted into the body fluids and which directly recognize an antigen. The cellular system, in contrast, relies on special cells which recognize and kill other cells which are producing foreign antigens. This basic functional division reflects two different strategies of immune defense. Humoral immunity is mainly directed at antigens which are exogenous to the host's somatic cells or on the surface of cells whereas the cellular system responds to antigens which are actively synthesized within cells or derived from phagocytosed exogenous antigens.

Antibody molecules, the effectors of humoral immunity, are secreted by special B lymphoid cells, B cells, in response to antigen, co-receptor stimulation and cytokines. Antibodies can bind to and inactivate antigen directly (neutralizing antibodies) or activate other cells of the immune system to destroy the antigen depending on isotype; IgM, IgG1, etc. Isotype class switching in B cells is controlled by cytokine milieu. Abnormalities in antigen response, co-receptor engagement or cytokine milieu can lead to suboptimal immune responses tolerance or autoimmunity.

Cellular immune recognition is mediated by a special class of lymphoid cells, the T cells. These cells do not recognize whole antigens but instead respond to degraded peptide fragments thereof which appear on the surface of the antigen presenting cells bound to surface proteins called major histocompatibility complex (MHC) molecules. Two subgroups of T cells exist, the CD4 T cells recognize peptide fragments bound to MHC class II molecules while CD8 T cells recognize peptide fragments bound to MHC class I molecules. CD8 T cells include the population of cytotoxic T cells (CTL) able to specifically lyse antigen presenting cells. Essentially all nucleated cells have class I molecules. It is believed that proteins produced within the cell are continually degraded to peptides as part of normal cellular metabolism. These fragments are bound to the MHC molecules and are transported to the cell surface. Thus the cellular immune system is constantly monitoring the spectra of proteins produced in all cells in the body and is poised to eliminate any cells producing foreign antigens or abnormal self antigens. CD4 T cells recognize mainly exogenous antigens that were taken up by antigen processing cells wherein the antigen is degraded and appears as peptide fragment on class II MHC molecules. The effector function of CD4 cells is primarily regulation of immune responses by release of cytokines. According to the cytokine profiles secreted, two subclasses of CD4 cells have been defined, the TH1 and TH2 cells. It is believed that in various infections and allergic and autoimmune diseases the type of the CD4 T cell subclass activated (TH1 vs. TH2) critically influences the outcome of the immune response.

Vaccination is the process of preparing an animal to respond to an antigen. Vaccination is more complex than immune recognition and involves not only B cells and cytotoxic T cells but other types of lymphoid cells as well. During vaccination, cells which recognize the antigen (B cells or T cells) are clonally expanded. In addition, the population of ancillary cells (helper T cells which provide co-receptor and cytokine stimulation) specific for the antigen also increase.

Vaccination also involves specialized antigen presenting cells which can process the antigen and display it in a form which can stimulate one of the two pathways (macrophages and dendritic cells).

A foreign antigen is introduced into an animal where it activates specific B cells by binding to surface immunoglobulins. It is also taken up by antigen processing cells, wherein it is degraded, and appears in fragments on the surface of these cells bound to Class II MHC molecules. Peptides bound to class II molecules are capable of stimulating the helper class of T cells (CD4 T cells). Both helper T cells and activated B cells are required to produce active humoral immunization. Cellular immunity is stimulated by a similar mechanism but entry into the MHC I presentation pathway of antigen presenting cells is typically by intracellular pathogen replication and not achieved by injection of protein antigen only.

Standard vaccination schemes nearly always produce a humoral immune response. The humoral system protects a vaccinated individual from subsequent challenge from a pathogen and can prevent the spread of an intracellular infection if the pathogen goes through an extracellular phase during its life cycle; however, it can do relatively little to eliminate intracellular pathogens. Cytotoxic immunity complements the humoral system by eliminating the infected cells and cancer cells. Thus effective vaccination should activate both types of immunity.

A cytotoxic T cell response is necessary to remove intracellular pathogens such as viruses as well as malignant cells. It has proven difficult to present an exogenously administered antigen in adequate concentrations in conjunction with Class I molecules to assure an adequate response. This has severely hindered the development of vaccines against tumor-specific antigens (e.g., on breast or colon cancer cells), and against weakly immunogenic viral proteins (e.g., HIV, Herpes, non-A, non-B hepatitis, CMV and EBV). It would be desirable to provide a cellular immune response alone in immunizing against agents such as viruses for which antibodies have been shown to enhance infectivity. It would also be useful to provide such a response against both chronic and latent viral infections and against malignant cells.

Directed and elevated immune responses to antigens can be achieved by the use of adjuvants and/or delivery vehicles. The term "immune adjuvant" refers to compounds which when administered to an individual or tested in vitro, increase the immune response to an antigen. Some antigens are weakly immunogenic when administered alone or are toxic at the concentration which evokes immune responses. An immune adjuvant may enhance the immune response of the individual to the antigen by making the antigen more immunogenic. The adjuvant effect may also lower the dose of antigen necessary to achieve an immune response by enhancing presentation, influence the cytokine milieu or alter co-receptor expression on antigen presenting cells.

In the pharmaceutical compositions of the present invention liposomes can serve as carriers for the direction of antigen to antigen presenting cells. It has been demonstrated that liposomes can serve to heighten some humoral immune responses and to provide exogenously administered antigen a vehicle for entry into the MHC class I presentation pathway thus allowing the stimulation of cytotoxic T cells.

The present invention relates to a pharmaceutical composition comprising at least one fragment of a polynucleotide and at least one antigen. The antigen can be an endogenous antigen of the host, in that case exogenous addition of antigen to the pharmaceutical composition may not be required.

The term "polynucleotide" in the sense of the present invention comprises all types of polynucleotides, e.g. RNA or DNA whereby, however, DNA polynucleotides are preferred.

The term antigen in the sense of the present invention means a molecule that can elicit an immune response. The immune response may be either humoral, i.e. antibody-mediated, or cellular, i.e. cell-mediated. An antigen that evokes an immune response is commonly referred to as immunogen. Generally only foreign or "non-self" molecules are immunogenic. It should be understood, however, that in the sense of the present invention the term antigen comprises also certain "self" molecules such as tumor cells, tumor markers or self antigens in autoimmunity. Those compounds may not be foreign to the host to be treated, but may be comprised under the term antigen, since sometimes an immune response to self molecules is desired.

Usually the larger and more complex a molecule is, the more immunogenic sites it will have. A single antigen may contain many epitopes which are specific areas of the molecule with a three-dimensional configuration that induces an immune response. Complex molecules, such as large proteins composed of many different amino acids contain more epitopes than a comparatively simple polysaccharide composed of two or three monosaccharide repeats. The immune response to a given antigen can vary greatly among species and individuals within a species due to immune regulation genes. The pharmaceutical compositions of the present invention comprise therefore specific polynucleotides which provoke a suitable immune response.

In a preferred embodiment of the present invention the polynucleotide is a DNA oligonucleotide. DNA oligonucleotides as used in the present invention are preferably short fragments of a DNA having about 100, preferably five to about 40 and more, preferably 15 to about 25 nucleotides.

The DNA oligomers provide when applied in relatively low quantities the effect of an immune adjuvant and immunomodulatory substance together with low toxicity and low side effects. For example can a combination of a suitable DNA oligomer and tumor cells or specific tumor markers induce tumor regression.

In a preferred embodiment of the present invention the DNA oligonucleotide is single-stranded or double-stranded.

whereby single-stranded DNA is especially preferred.

It is especially preferred that the polynucleotide as used in the present invention comprises the sequence of a binding site for transcription factors or parts thereof or that the sequence of the polynucleotide is complementary to said binding site for transcription factors or a part thereof.

In the course of the present invention it has been found that polynucleotides are preferred which have a sequence corresponding to the binding site of transcription factors or which are complementary thereto. Generally it is sufficient that at least a part of said binding site is contained within the polynucleotides.

The expression of individual genes is a rather complex process. These processes are mediated by several specific regulatory DNA regions found in the promotor regions of almost all genes. These regulatory sequences are frequently referred to as response elements. They are binding sites for sequence-specific DNA binding proteins which are called transcription factors. Some transcription factors are general purpose factors (basal transcription factors) required for transcription of all genes while other act on specific genes or classes of genes by binding in a sequence-specific manner to response elements and other sequence motives within the corresponding gene promoters. The expression of many of these transcription factors is developmentally and also tissue-specifically controlled and is itself subject to the action of other transcription factors and other accessory proteins such as nuclear receptors. Binding sites for transcription factors are often clustered and a variety of transcription factors have been found to form complexes with others or to compete with others for binding to overlapping DNA-binding motives. Several structural motives have been found within those regions of transcription factor proteins recognizing and contacting DNA. Within each of these structural motives there are often families of related proteins that recognize similar DNA sequences and are conserved throughout the eukaryotic kingdom.

In a preferred embodiment of the present invention the sequences of binding sites for transcription factors of cytokines are especially preferred.

In another preferred embodiment of the present invention the polynucleotide of a pharmaceutical composition comprises the sequence 5'PuPuCGPyC wherein Pu means purine and comprises adenine and guanine and Py has the meaning of pyrimidine and comprises cytosine, thymine and uracil and wherein A means adenine, C means cytosine and G means guanine.

Those preferred sequences of the present invention differ slightly from the CpG motives as disclosed in the prior art.

It is preferred that the polynucleotide comprises at least one phosphorothioate linkage. In the preferred phosphorothioate derivatives of the polynucleotides at least one oxygen atom of the phosphate backbone of the polynucleotide is replaced by a sulphur atom. Those compounds are more stable against degradation.

It is also possible to add to the pharmaceutical composition of the present invention further classical adjuvants which are known to the person skilled in the art. Examples may be preparations from the cell walls of bacteria. The pharmaceutical compositions of the present invention comprise also additives known to the person skilled in the art depending on the administrative way e.g. oral, parenteral or rectal.

It is possible to use within the scope of the present invention a wide variety of antigens. A preferred antigen is selected from the group comprising peptides, polypeptides, steroids and tumor cells.

Examples for such antigens may be killed intact bacteria, toxoids (i.e. toxins that are still immunogenic but are rendered biologically inactive by treatment with a chemical, heat or mutation), subunit vaccines in which only the non-toxic portion of the molecule is used or life-attenuated vaccines in which a viral or bacterial strain is rendered non-pathogenic (e.g. by passaging the virus in cell culture or deletion of bacterial genes), but is still able to multiply to a limited degree thereby eliciting protective immune response in the absence of disease symptoms.

Since the pharmaceutical composition of the present invention can also be used for the treatment and/or prophylaxis of such diseases which are not caused by foreign organisms the antigen may be also an antigen of the own body like for example a tumor antigen. For the treatment of autoimmune diseases or in order to influence positively the tolerance it may also be effective to use antigens derived from the body to be treated. In that cases it may not be required to add the antigen to the pharmaceutical composition since the antigen is already present in the host.

In the accomplishment of the foregoing objectives of the invention, vaccine formulations are made which induce both humoral and cellular immune responses to antigen using sequences with the motif 5'Pu-Pu-CpG-Py-Py-3'. Some sequences, especially with the motif 5'Pu-Pu-CpG-Py-Py-3' can be toxic and lethal. It is therefore another aspect of the invention to modify these sequences in such a way that immune adjuvant activity is maintained but toxicity is eliminated. In addition, DNA sequences are described which do not follow this motif but serve as immune adjuvant and immune response modifiers. A common aspect of the invention is that sequences from eukaryotic promoters are used. ssDNA sequences containing palindromic and non-palindromic transcription response elements serve also as immune adjuvant or immune response modifiers, that is sequences recognized by transcription factors (proteins which regulate gene transcription). These sequences are capable of modulating lymphocyte cell surface markers and cytokine release in vitro and in vivo. In yet another aspect of the invention, the method is utilized as a therapeutic approach for treating immune system tolerance and control of tumor.

The teaching of the present invention can be used for modulating the immune response to antigen by using certain DNA oligomers. The particular DNA depends on the desired outcome. The invention works in vitro and in vivo in warm blooded animals.

A common aspect of the invention is to use certain DNA oligonucleotides to specifically influence the regulation and signalling machinery of eukaryotic cells. In particular, growth, induction of cellular transcription and translation, protein synthesis or protein secretion can be modified by DNA oligonucleotides. In addition, response pattern of eukaryotic cells towards exogenous stimuli are subject of modification. Modification of the eukaryotic cell's response is controlled by the sequence of the oligonucleotide, i.e. is sequence-specific. The invention gives a rule how active DNA oligonucleotides can be selected. Accordingly, active DNA oligonucleotides are derived from DNA-sequences able to be bound by transcription factors. These sequences can be identified from eukaryotic promoters.

The invention concerns also the use of specific DNA oligos for the preparation of a pharmaceutical composition which have the effect to interfere, to modulate and to regulate responses of the innate and acquired immune system. Those include enhancement of immune responses (including vaccination), modulation of immune responses and suppression of immune responses.

Use of DNA oligomers to enhance the reactivity of immune cells to viral, bacterial and parasitic antigens is claimed. Enhancement includes induction of immunological memory, cytotoxic T cells, cytokine release and augmentation of innate immunity (phagocytosis, cytokine release and cytolytic function). In particular, DNA oligomers can be used as an adjuvants for T- and B-cell vaccination. Enhancement further includes induction of reactivity against weak or tumor antigens. The use of DNA oligos to break tolerance in anergic T and B cells e.g. against tumor antigens is further claimed. The claim incorporates the use of DNA oligos as adjuvants in vaccination against tumor-defined antigens and immunostimulatory substances in an ongoing immune response against tumors.

Use of DNA oligos to modulate responses of the acquired immune system is also an aspect of the present invention. Desired immune responses can be stimulated while adverse effects can be suppressed by DNA oligos. DNA oligos can shift an immune response to a TH1-type or a TH2-type of reactivity. This effect modulates the response during parasitic infections (Leishmaniasis, Toxoplasmosis, or Mycobacteriosis). In addition, the use of DNA oligos to direct a B cell immune response towards certain classes of immunoglobulins, thus bypassing and overcoming the adverse Ig-dependent diseases like Ig-E-mediated allergy is also an aspect of the present invention.

Use of DNA oligos to suppress immune reactions of the innate and acquired immune system is also an aspect of the present invention. DNA oligos can be used to suppress B- and T-cell responses towards transplantation antigens and thus induce transplantation tolerance. DNA oligos can further be used to suppress ongoing or manifested immune responses as it is the case during T- and B-cell dependent autoimmune diseases.

The pharmaceutical preparations of the present invention preferably comprise polynucleotides applied together with antigen either in free form or entrapped in liposomes. The science of forming liposomes is now well developed. Liposomes are unilamellar or multilamellar vesicles having a membrane portion formed of lipophilic material and an interior aqueous portion. The aqueous portion is used in the present invention to contain the protein material to be delivered to the antigen presenting cell. Conventional methods can be used to prepare liposomes. They are taken up by macrophages and dendritic cells in vivo and are thus particularly effective for delivery of antigen to these cells.

Liposomes were manufactured by a rehydration entrapment method. Preferably the liposomes were prepared as follows. 18.0 mg phosphatidylcholine; 2.0 mg phosphatidylglycerol and 5.0 mg cholesterol, a 2:0.2:1 ratio, was suspended in 5.0 ml chloroform in a 100 ml round bottom flask. The mixture was rotary evaporated under reduced pressure until a thin lipid film formed on the flask wall. Residual chloroform was removed by vacuum desiccation. 3 mg of ovalbumin was solubilized in 1.0 ml of PBS. This solution was slowly added to the dried lipid and hand-shaken until the lipids were resuspended. The crude protein liposome mixture was allowed to equilibrate for 30 min at room temperature, transferred to a microfuge tube and centrifuged at 6,000 rpm for 5 min in an Eppendorf® microfuge. The mixture was then filter-extruded through a 0.2 µm Anotop10® syringe mount filter. To this mixture 10 nmol oligomer was added per 100 µl.

In the experiments mice were usually immunized by way of the hind footpad with 50 µl per foot of peptide liposome preparation. After four days, the draining popliteal lymph nodes (LN) were removed and a single-cell suspension was prepared. The cells were cultured for four days in the presence of IL-2 and a chromium release assay was performed utilizing the syngenic target cell EL-4 or the cell line EG-7 which is transfected with the gene for ovalbumin and thus presents ovalbumin peptides as antigen (fig. 1). In some experiments EL-4 pulsed with the MHC class I (Kb) restricted ovalbumin peptide SIINFEKL was used as the target for kill.

The invention is further illustrated in the following figures:

Figure 1 shows cytolytic T cell induction in vivo with different ssDNA adjuvants. Mice were injected with the antigen ovalbumin entrapped in liposomes using the indicated adjuvant. T cells were harvested from the mice and tested for the specific recognition of the antigen. The assay indicated a strong activation of the cellular immune system due to the adjuvant. AP-1, Stat-5/6, SIE, cMyb, Stat3, Stat4, Stat5, SP-1, C/EBP, IL-13, Stat1, CRE, Erg and IRF-

1 are binding sites for transcription factors.

Figure 2 shows a summary of cytolytic T cell assay, different sequences were tested. In vivo induced CTL tested for specific antigen recognition. Data are expressed as lytic units. A lytic unit is arbitrarily defined as the number of lymphocytes required to yield 30% specific lysis. The number is the lytic units per 10^6 effector cells. Lytic units are a way to compare cytolytic T cell populations.

Figure 3 shows antibody production by ssDNA adjuvants post injection. Endpoint antibody titer assay: Mice were injected with the antigen ovalbumin entrapped in liposomes using either no adjuvant or a TRE adjuvant. The mice were boosted once. The assay indicates a strong adjuvant effect for the enhancement of antibody production in response to injected antigen. The adjuvant-assisted increase in response is particularly strong for IgG2a and IgG2b. Of note is the differential induction of antibody isotype dependent on the adjuvant used indicating differential cytokine release.

Figure 4 shows cytokine induction in vivo with transcription response element ssDNA adjuvant CRE. The cytokine release pattern induced by the transcription response element CRE. Mice were injected with the ssDNA and at the indicated time serum was sampled and cytokine release measured.

Figure 5 shows phenotype changes (IL-2 receptor expression) of B and T cells in vivo with different transcription response element ssDNA adjuvants. Analysis of a relevant cell surface marker after treatment with ssDNA. The IL-2 receptor binds and transduces a proliferation signal from IL-2 to cells of the immune system. The TRE (transcription regulatory elements) sequences vary in their stimulatory capacity for inducing IL-2 receptor expression. Some TRE are inhibitory indicating a potential use for negative immunomodulation.

Figure 6 shows tumor regression and control with ssDNA. ssDNA induces regression of prexistant tumor. Mice were injected with tumorigenic numbers of a syngenic tumor cell. Four days after the challenge the mice were treated with ssDNA alone or ssDNA plus a sub-tumorigenic number of tumor cells, which served as an antigen source. The progression of tumor growth was significantly controlled. Five mice were included per group.

EXAMPLE 1

Bacterially derived sequences can be used as an adjuvant for cytolytic T cell activation in vivo.

Three sequences containing the sequence motif of 5'Pu-Pu-CpG-Py-Py-3' are described in the literature for having immunostimulating properties. One sequence is derived from the ampicillin resistance gene of *E. coli*, here termed AMP (TCATTGGAAACGTTCTTCGGGCG). The second sequence is derived from a BCG gene and is termed BCG-A4A (ACCGATGACGTCGCCGGTGACGGCACCACG). The third is a synthetic sequence claimed to be a prototype of bacterial CpG sequences, referred to by Krieg et.al. as 1668 (TCCATGACGTTCTGATGCT). These sequences were synthesized to include a phosphorothioate linkage to reduce destruction by DNase. These oligomers served as an adjuvant in combination with ovalbumin to induce a cytolytic T cell response.

EXAMPLE 2

Bacterial CpG containing sequences may be toxic.

We observed that the above-described oligomers could be highly toxic in vivo for mice sensitive to TNF- α . The 1668 oligomer was previously described by Krieg and co-workers for its ability to induce murine B cell proliferation, induce IL-6 release from B cells and induce INF- γ release from B cells and induce INF- γ release from NK1.1 cells. We found in addition that 1668 and AMP were highly lethal in mice sensitized to the effects of TNF- α (Table 1). However, under certain circumstances these sequences may be useful therapeutically. One could foresee the use of these sequences once the risk is properly assessed.

TABLE 1

Death due to lethal shock	
1668 + D-gal	5/5
1668 + LPS	3/3
AMP + LPS	2/3

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TABLE 1 (continued)

Death due to lethal shock	
Control	0/3
Ratio= mice killed/mice injected	

5

10 For lethal shock, Balb/c mice were injected intraperitoneally with 10 nmol 1668 in 200 μ l PBS plus 20 mg D-galactosamine in 200 μ l PBS. Alternatively mice were injected intravenously with 10 nmol 1668, AMP or PBS followed at four hours with 50 μ g LPS.

EXAMPLE 3

15 Use of eukaryotic transcription regulatory elements or sequence manipulation prevents toxic shock symptoms. Due to toxicity, the need is established for the discovery of non-toxic sequences for safe human and animal use. Since toxicity is at issue when developing vaccine adjuvants and therapeutics, we were interested to develop oligomers that circumvented toxicity but retained immunostimulatory properties. We screened eukaryotic sequences displaying the absence of lethality but maintaining immunostimulatory qualities. One such sequence was the cyclic AMP response element (CRE) which is the consensus binding site for the transcription factors CREB/ATF as well as the AP-1 family, sequence (GATTGCCTGACGTCAGAGAG) [Roesler, W. J. et al., J. Biol. Chem. 263, 9063-9066 (1988)]. Table 2 demonstrates the loss of lethality of the CRE sequence. To further evaluate the sequence specificity of these effects we made sequence exchanges between CRE and 1668. An exchange of only two nucleotides between CRE and 1668 resulted in a loss of lethality (Table 2).

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Table 2 Sequences of oligomers and death due to lethal shock

a		
1668	TCCATGACGTTCTGATGCT	
30 CRE	ATTGCCTGACGTCAGAGAGC	
1668-CA	TCCATGACGTCAGTATGATGCT	
CRE-TC	ATTGCCTGACGTTCTGAGAGC	
b		
35 1668	5/5	
CRE	0/5	
1668-CA	0/3	
CRE-TC	3/3	

40

Lethality was determined as in example 2. The 1668 sequence fortuitously contains a combination transcription response elements, that transcription factor binding sites (TGACGTTCC). This element represents the binding site for HSVIP04 (ATF), HSINS04 (CREB half site), CAMV35SR03 (HBP-1a yeast) or ADE422 (AP-1) in combination with an HSIL606 site which is a repressor site (sequence analysis from EMBL database Heidelberg). This sequence can be found in the 5' non-coding regions (promoters) of several eukaryotic cytokine genes including human IL-13 promoter and IL-12 p40 intron 1. The CRE sequence contains all the response elements cited above except for HSIL606 and it contains the full CRE palindromic sequence (TGACGTCA). In accordance with the invention, the CRE sequence did not induce death and changes in the 1668 eliminate toxicity.

50 TNF- α release is a hallmark of lethal toxic shock [Tracey, K. J. et al., Science 234, 470-474 (1986), Tracey, K. J. et al., Nature 330, 662-664 (1987)].

An exchange of only two nucleotides between CRE and 1668 resulted in a loss of macrophage induced TNF- α release activity, the sequence is given in Table 2. The reported 6-mer active core sequence of 1668 contains the CpG flanked by two 5' purines and two 3' pyrimidines and the exchange of CA for TC does not affect this motif, however, TNF- α release was severely diminished. Thus the broader core 8-mer sequence or the transcription response element and not the surrounding sequence environment was responsible for these effects. In accordance with the invention, when utilizing macrophage derived TNF- α release as a marker, the 5'Pu-Pu-CpG-Py-Py-3' motif was not satisfactory for pre-

dicting oligomer activity or toxicity. Additionally, in contrast to 1668, CRE did not induce IL-6 release in vivo or from the ANA-1 cell line in vitro.

EXAMPLE 4

ssDNA containing transcription response elements (TRE) serve as adjuvant for antibody production.

In accordance with the invention, eukaryotic transcription response elements relevant to the immune system serve as immune adjuvant. To test the adjuvant qualities of different sequences we injected mice with either free ovalbumin plus oligomer or liposome encapsulated ovalbumin plus oligomer. The mice were boosted at day 14 and after one week

Figure 3 shows that different sequences strongly potentiated the antibody response and induced class switching toward IgG1, IgG2a and IgG2b.

Liposomes containing ovalbumin were prepared as described above. For antibody induction, 300 µg ovalbumin in PBS or liposomes containing ovalbumin were injected +/- 10 nmol oligomer in the hind footpads of C57/B6 mice. A boost of the like inoculum was given at two weeks and one week later blood was extracted for serum antibody titering.

EXAMPLE 5

In accordance with the invention, ssDNA containing transcription response elements serve as adjuvant for cellular immunity.

We have described the use of liposomes in combination with Quil A or QS-21 to induce cytolytic T cells (CTL) to either soluble antigen or peptides [Lipford, G. B., Wagner, H. & Heeg, K., Vaccine 12, 73-80 (1994), Lipford, G. B. et al., J. Immunol. 150, 1212-1222 (1993)]. Liposome entrapped antigen alone was an ineffective inducer of CTL activity, but with the addition of immunostimulatory saponins the inoculum became effective. To test the in vivo T cell immunomodulatory potential of oligomers we utilized this vehicle to demonstrate primary activation of CTL. Figure 1 shows a substantial primary CTL response induced by an inoculum of ovalbumin liposomes plus ssDNA matching transcription response elements. The lytic units value interpolated from these curves was approximately 500 L.U. as compared to <20 L.U. for ovalbumin liposomes only (Table 3). CTL memory, an important quality for vaccine protection, could also be demonstrated with these inoculum. If mice were rested for two weeks after the first injection and reinjected with the same inoculum, CRE recalled CTL displaying lytic units measured at approximately 1500 L.U. (Table 3). Additional, when the inoculum was formulated with the immunodominant K^b restricted ovalbumin peptide SIINFEKL, the oligomers induced a specific primary CTL response. Thus oligomers serve as a strong in vivo stimulus resulting in T cell activation and the proliferation of antigen specific CTL effectors. The inoculum can contain protein or peptide as the target antigen.

Table 3

Cytolytic T cell response induced by oligomer in lytic units		
	CRE	PBS
Primary CTL	526 L.U.	<20 L.U.
Secondary CTL	1555 L.U.	<20 L.U.

Several other sequences have been determined to have immunomodulatory effects. Table 4 list tested eukaryotic transcription response elements (TRE), which are preferably used in the present invention.

Table 4

Sequences of eukaryotic TRE tested	
CRE	GATTGCCTGACGTCAGAGAG
IL-13	GGAATGACGTTCCCTGTG
IL-12p40	AGCTATGACGTTCCAAGG
AP-1	GCTTGATGACTCAGCCGGAA

Table 4 (continued)

Sequences of eukaryotic TRE tested	
SP1	TCGATCGGGGCGGGGCGAGC
C/EBP	TGCAGATTGCGCAATCTGCA
EGR	AGCGGGGGCGAGCGGGGGCG
GAS/ISRE	TACTTTCAGTTTCATATTACTCTA
SIE	GTCCATTTCCTGTAAGTG
STAT1	TATGCATATTCCTGTAAGTG
STAT3	GATCCTTCTGGGAATTCCTA
STAT4	CTGATTTCCCCCAAATGATG
STAT5	AGATTTCTAGGAATCAATC
STAT5/6	GTATTTCCAGAAAAGGAAC
IRF-1	AAGCGAAAATGAAATTGACT
c-Myb	CAGGCATAACGGTCCGTAG
NFkB	ATATAGGGGAAATTCAGC
HSINF	CAAAAAAATTCAGTCCTT
HSIL-6	ATGTTTCTCGCTGCCAG
CRENFkB	CTCTGACGTCAGGGAAATTCAGC

The relative strength of the various transcription response elements for adjuvant potential for CTL induction can be seen in fig. 2.

Example 6

In accordance with the invention, ssDNA containing transcription response elements induce cytokine release in vivo.

Mice were injected with formulations containing different oligomers plus liposome or liposomes containing 300 µg ovalbumin. Serum was collected at various times and analyzed for serum cytokine levels by specific ELISA.

In addition T cells produce IL-2 in response to ssDNA. Thus in accordance with the invention, cytokine release patterns are sequence dependent and thus the eukaryotic transcription response element used influences the cytokine release outcome and thus biological effect. This aspect of the invention is highly relevant, because cytokine release patterns can be influenced by different transcription response elements. ssDNA can be used to induce cytokine release in vivo to produce a desired outcome. This outcome could be to produce immune enhancement or immune suppression.

In accordance with the invention, ssDNA containing transcription response elements can break tolerance in T cells. It can be demonstrated that T cells induced to become tolerant (that is non-response to antigen signals) in vivo by SEB injection break tolerance if ssDNA is injected up to seven days post tolerance induction. This finding has relevance for the development of specific modulations for desired biological outcome. It can be foreseen that ssDNA can be used to elevate the immune response of immunocompromised patients.

EXAMPLE 7

In accordance with the invention, ssDNA containing transcription response elements induce co-receptor and surface receptor change on B and T cells. This finding has relevance for the development of specific modulations for desired biological outcome.

Mouse spleen cells were harvested and cultured 24 hours in the presence of the list eukaryotic TRE. Cell surface markers were measured on T or B cell by FACS analysis.

Some of the transcription response elements have a positive effect and some have a negative effect. Both outcome are of potential use. If immune enhancement is desired a sequence inducing a given cell surface marker would be of use. If immune suppression is desired a sequence suppressing a given cell surface marker would be of use.

EXAMPLE 8

In accordance with the invention, ssDNA containing transcription response elements can induce tumor control or regression.

Figure 6 demonstrates that mice challenged with tumor cells progress rapidly to display measurable subcutaneous tumor. These tumor are lethal. If ssDNA is injected four days post challenge the tumor regress or show retarded rates of growth. In addition if cells are provided as antigen the same observation is made.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

APPLICANT:

- (A) NAME: Lipford/Wagner/Heeg
- (B) STREET:
- (C) CITY:
- (E) COUNTRY: Germany
- (F) POSTAL CODE (ZIP): 81675

TITLE OF INVENTION:

Pharmaceutical composition comprising
a polynucleotide and an antigen
especially for vaccination

NUMBER OF SEQUENCES: 28

COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PADAT Sequenzmodul, Version 1.0

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

RRCGYC

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TCATTGGAAA ACGTTCTTCG GGGC

24

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ACCGATGACG TCGCCGGTGA CGGCACCACG

30

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TCCATGACGT TCCTGATGCT

20

(2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TCCATGACGT TCCTGATGCT

20

(2) INFORMATION FOR SEQ ID NO: 6:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

ATTGCCTGAC GTCAGAGAGC

20

(2) INFORMATION FOR SEQ ID NO: 7:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TCCATGACGT CACTGATGCT

20

(2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

ATTGCCTGAC GTTCGAGAGC

20

(2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GATTGCCTGA CGTCAGAGAG

20

(2) INFORMATION FOR SEQ ID NO: 10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: Genomic DNA

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GGAATGACGT TCCCTGTG

18

(2) INFORMATION FOR SEQ ID NO: 11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: Genomic DNA

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

AGCTATGACG TTCCAAGG

18

(2) INFORMATION FOR SEQ ID NO: 12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: Genomic DNA

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GCTTGATGAC TCAGCCGGAA

20

(2) INFORMATION FOR SEQ ID NO: 13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 bas pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

TCGATCGGGG CGGGGCGAGC

20

(2) INFORMATION FOR SEQ ID NO: 14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

TGCAGATTGC GCAATCTGCA

20

(2) INFORMATION FOR SEQ ID NO: 15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

AGCGGGGGCG AGCGGGGGCG

20

(2) INFORMATION FOR SEQ ID NO: 16:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 24 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

TACTTTCAGT TTCATATTAC TCTA

24

(2) INFORMATION FOR SEQ ID NO: 17:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GTCCATTTCC CGTAAATCTT

20

(2) INFORMATION FOR SEQ ID NO: 18:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

TATGCATATT CCTGTAAGTG

20

(2) INFORMATION FOR SEQ ID NO: 19:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

GATCCTTCTG GGAATTCCTA

20

(2) INFORMATION FOR SEQ ID NO: 20:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

CTGATTTCCT CGAAATGATG

20

(2) INFORMATION FOR SEQ ID NO: 21:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

AGATTCTAG GAATTCAATC

20

(2) INFORMATION FOR SEQ ID NO: 22:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

GTATTCCCA GAAAAGGAAC

20

(2) INFORMATION FOR SEQ ID NO: 23:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

AAGCGAAAAT GAAATTGACT

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(2) INFORMATION FOR SEQ ID NO: 24:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

CAGGCATAAC GGTTCCGTAG

20

(2) INFORMATION FOR SEQ ID NO: 25:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

ATATAGGGGA AATTCCAGC

20

(2) INFORMATION FOR SEQ ID NO: 26:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

CAAAAAAATT TCCAGTCCTT

20

(2) INFORMATION FOR SEQ ID NO: 27:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

ATGTTTTCCT GCGTTGCCAG

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(2) INFORMATION FOR SEQ ID NO: 28:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 26 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

CTCTGACGTC AGGGGAAATT TCCAGC

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Claims

1. Pharmaceutical composition comprising

- a) at least one fragment of a polynucleotide and
 b) at least one antigen.

2. Pharmaceutical composition according to claim 1 characterized in that the polynucleotide is a DNA oligonucleotide.

3. Pharmaceutical composition according to claim 2 characterized in that the DNA oligonucleotide is single stranded.

4. Pharmaceutical composition according to any of claims 1-3 characterized in that the polynucleotide has 5-40 nucleotides.

5. Pharmaceutical composition according to claim 4 characterized in that the polynucleotide has 15-25 nucleotides.

6. Pharmaceutical composition according to any of claims 1-5 characterized in that the polynucleotide (a) comprises the sequence of a binding site for transcription factors or a part thereof or the sequence of the polynucleotide is complementary to said binding site for transcription factors or a part thereof.

7. Pharmaceutical composition according to claims 1-5 characterized in that the polynucleotide comprises the sequence

5'PuPuCGPyC

wherein Pu means purine and comprises adenine and guanine and Py has the meaning of pyrimidine and comprises cytosine, thymine and uracil and wherein A means adenine, C means cytosine and G means guanine.

8. Pharmaceutical composition according to any of claims 1-7 characterized in that the polynucleotide comprises at least one phosphorothioate linkage.

9. Pharmaceutical composition according to any of the preceding claims characterized in that it comprises a further adjuvant.

10. Pharmaceutical composition according to any of the preceding claims characterized in that the antigen (b) is selected from the group comprising peptides, polypeptides, proteins, polysaccharides, steroids and tumor cells.

11. Pharmaceutical composition according to any of the preceding claims characterized in that the composition is a vaccine.

12. Pharmaceutical composition according to claim 11 characterized in that the vaccine is used for the treatment of cancer.

13. Pharmaceutical composition according to claim 11 characterized in that the vaccine is used for the prophylaxis and/or treatment of pathogen microorganisms.

5 14. Use of a polynucleotide for the preparation of a pharmaceutical composition for the modulation of immune response.

15. Use according to claim 14 wherein the modulation is a vaccination.

10 16. Use according to claim 14 wherein the modulation is selected from the group break of tolerance, regulation of TH1/TH2 helper cell responses, switch of Ig classes, treatment of autoimmune responses and induction of tolerances.

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Fig. 1

TRE Adjuvant: In Vivo CTL Induction Specific Lysis of Ovalbumin Target Cells

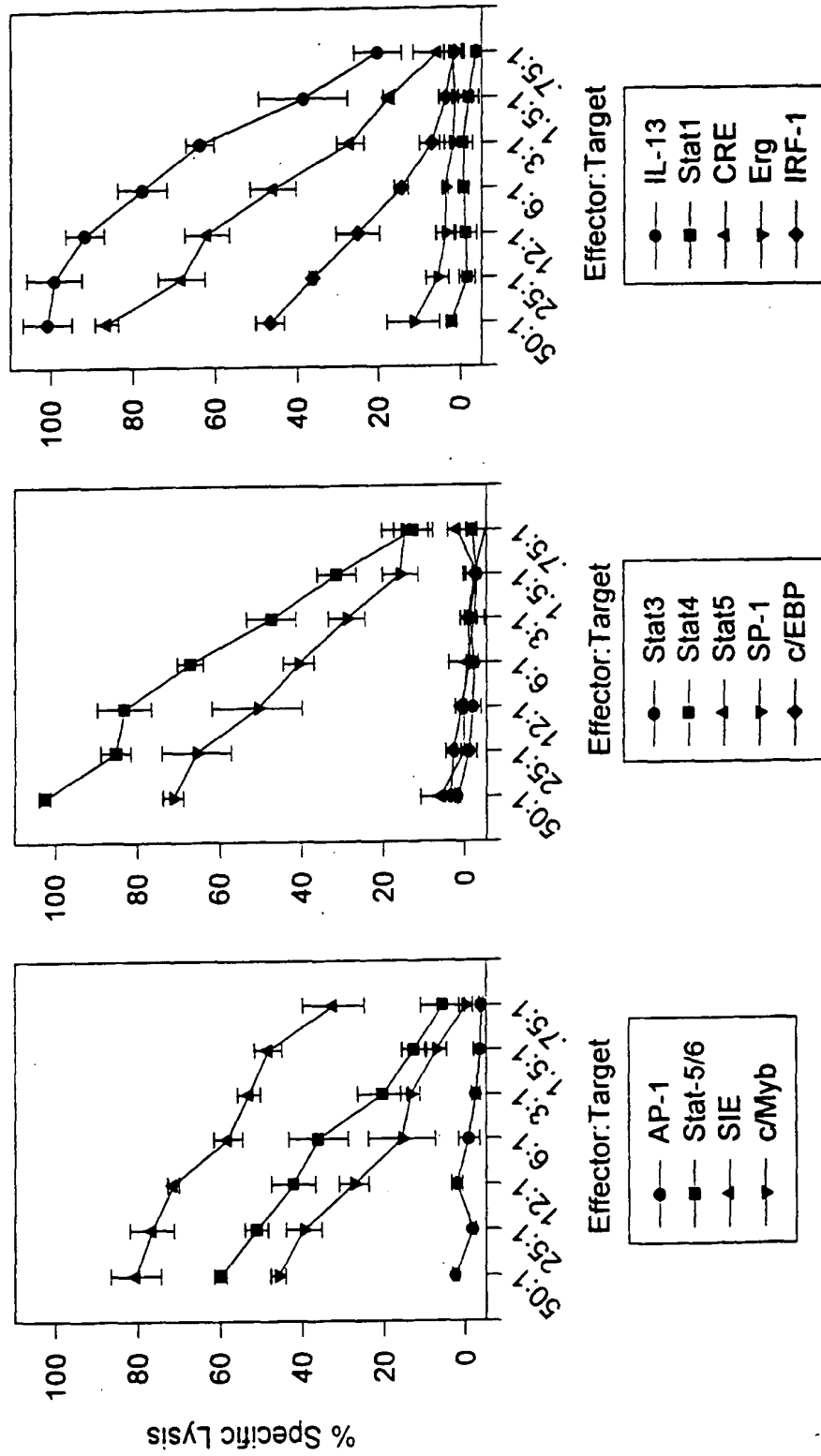


Fig. 2

TRE Adjuvant: In Vivo CTL Induction Specific Lysis in Lytic Units

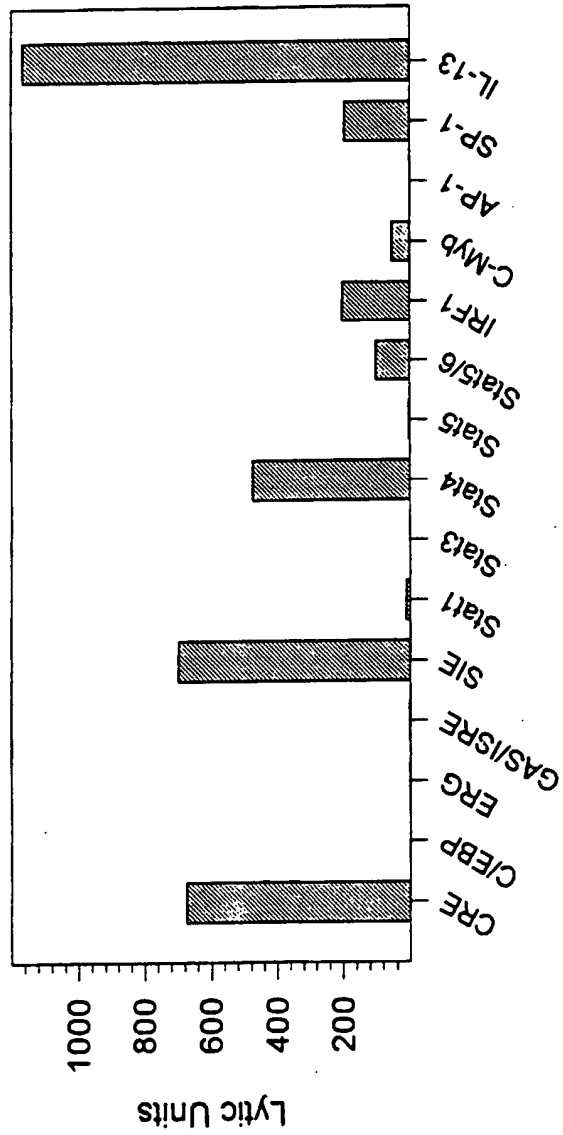


Fig. 3

TRE Adjuvant: In Vivo Antibody Induction
Specific Response to Antigen

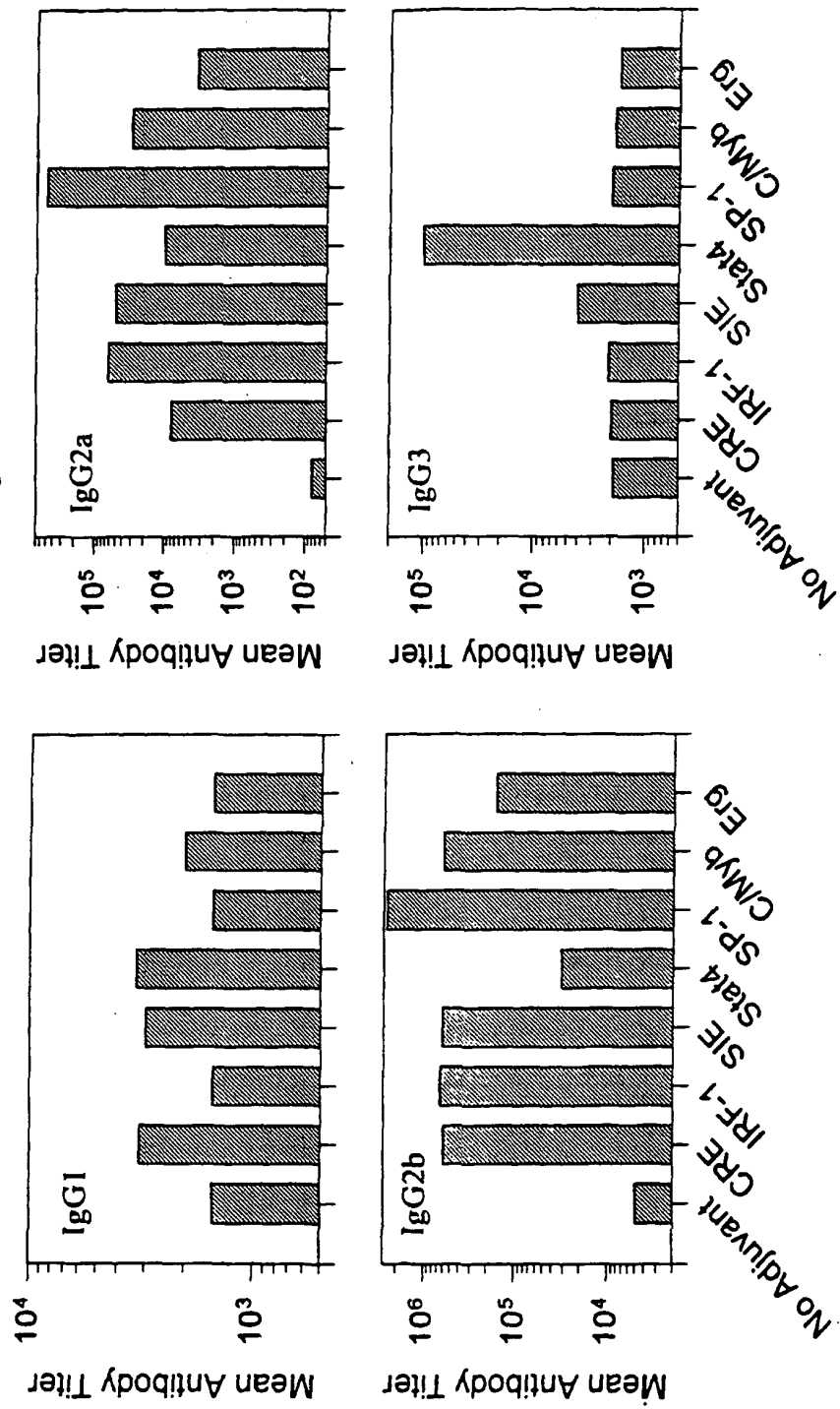
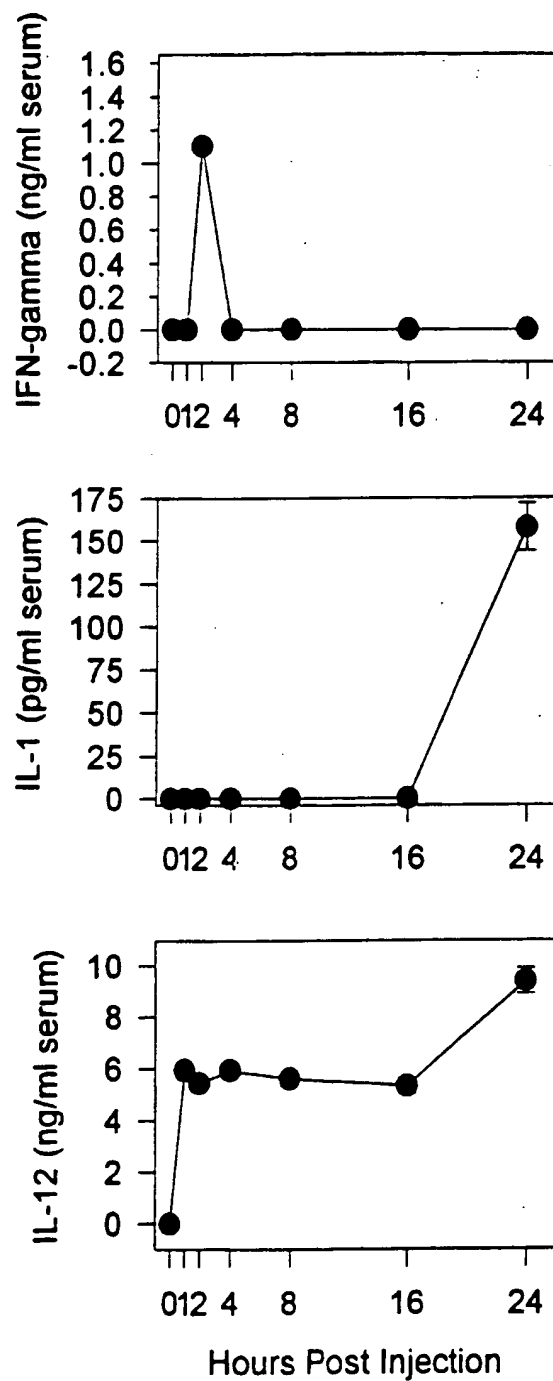


Fig. 4

CRE: In Vivo Cytokine Induction

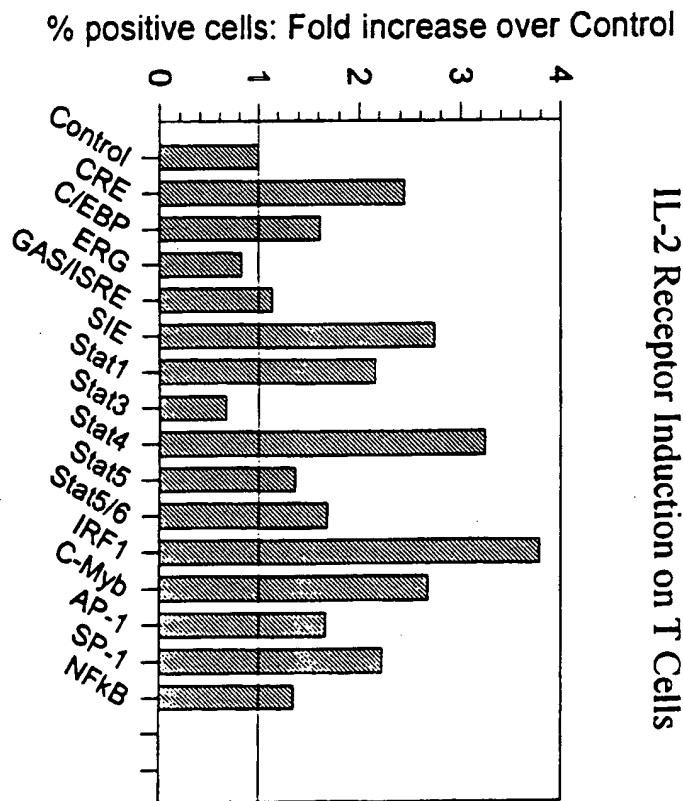
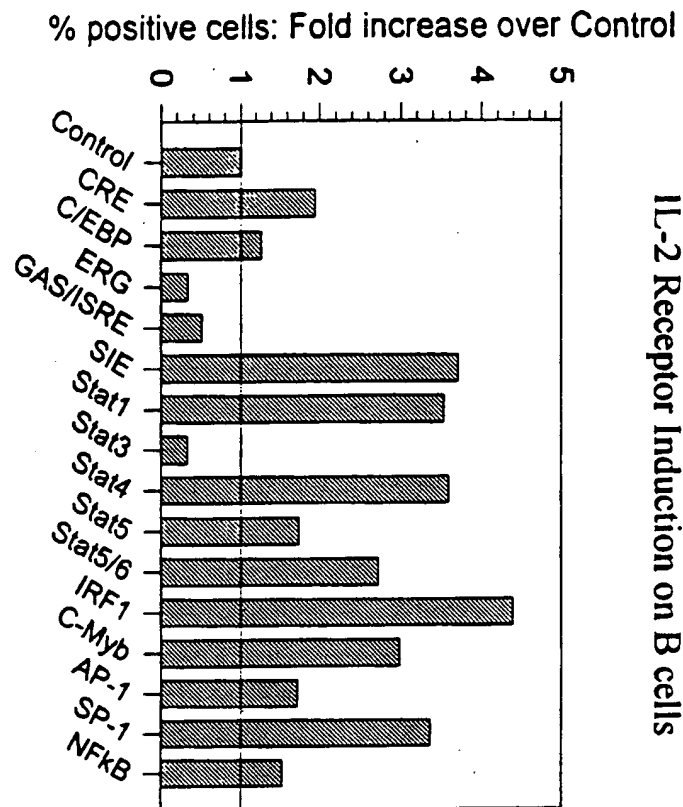
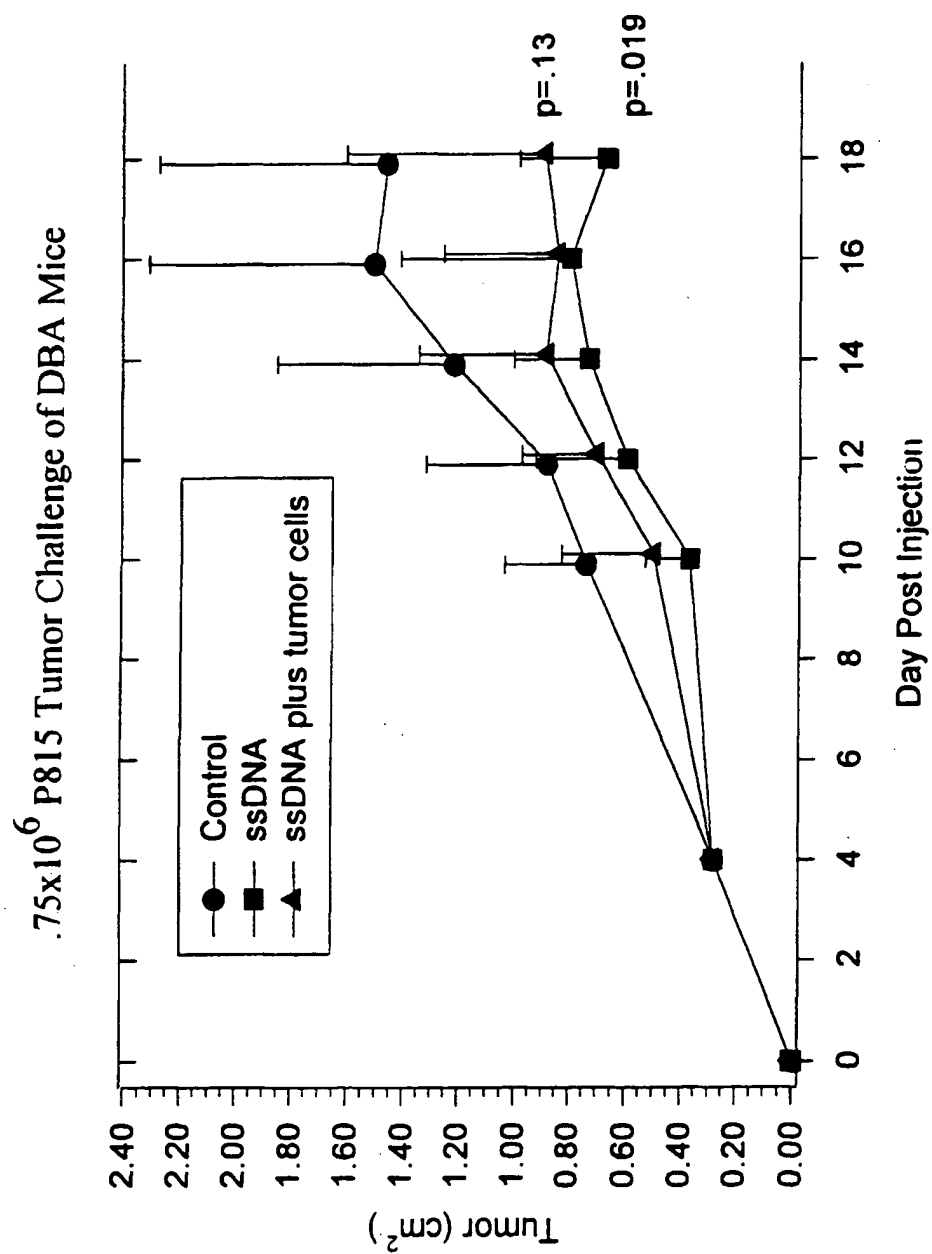


Fig. 5

Fig. 6





European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 97 10 1019

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X	WO 96 35782 A (APPLIED RESEARCH SYSTEMS ;SERLUP I CRESCENZI OTTAVIANO (IT); PEZZOT) 14 November 1996	1-6, 14-16	A61K39/39 A61K48/00 C07K14/195 C07K14/435
Y	* page 3, line 15 - page 5, line 11; claims 1-14 *	1-16	

X	US 3 906 092 A (HILLEMANN MAURICE R ET AL) 16 September 1975	1,2	
Y	* column 3, line 54 - column 5, line 36 *	1-16	

Y	WO 95 05853 A (UNIV CALIFORNIA ;CARSON DENNIS A (US); RAZ EYAL (US); HOWELL MERED) 2 March 1995	1-16	
	* claims 1-19,26,29 *		

A	BIOCHEMICAL PHARMACOLOGY 47 (1). 1994. 127-128. ISSN: 0006-2952, XP000569747 PETERSON M G ET AL: "Transcription factors: A new frontier in pharmaceutical development?."		
	* the whole document *		

A	US 5 328 987 A (MALISZEWSKI CHARLES R) 12 July 1994		
	* column 15, line 12 - line 19 *		

The present search report has been drawn up for all claims			
Place of search MUNICH		Date of completion of the search 23 July 1997	Examiner Halle, F
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons</p> <p>..... & : member of the same patent family, corresponding document</p>			

EPO FORM 1503 (01.92) (P04/C01)